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LEVEL 1 BIOASSAY SENSITIVITY

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16. ABSTRACT The report, based on a survey of published literature, establishes a set of sensitivity estimates for the Level 1 environmental assessment (EA) bioassays recommended by EPA's IERL-RTP. The estimates permit comparison of bioassay sensitivity for detecting toxicity or mutagenicity of pure chemicals and also estimation of the concentrations of toxic materials that could be in a mixture that registers negative in a particular test. Three tests--Ames Salmonella/microsome mutagenesis assay, in vitro rodent cell (CHO) clonal toxicity assay, and in vivo rodent toxicity assay--have substantial published data bases using study designs similar to those used in Level 1 EA tests. The report summarizes the sensitivity limits for detecting toxicity or mutagenicity for a variety of pure compounds with these three tests. Methods developed to assess these data will be applicable to other Level 1 tests such as the rabbit alveolar macrophage (RAM) assay and the aquatic and other ecological assays. However, these evaluations await the development of a sufficient data base with a wide variety of pure compounds. Two of the reviewed bioassays (Ames and CHO) were found to approach the sensitivity required of the Level 1 chemical analytical procedures.

17. KEY WORDS AND DOCUMENT ANALYSIS

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Bioassay	Stationary Sources	06A
Sensitivity	Level 1 Analysis	14G
Assessments	Environmental Assessment	14B
Toxicity		06T
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SUMMARY

Environmental assessment bioassays are conducted to detect toxic constituents in complex liquid, gaseous and solid waste emissions from industrial sites. Unlike instrumentation used in analytical chemistry, the power (limit of detectability) of the biological techniques to detect specific chemicals or even classes of chemicals is seldom known.

In this report, the published literature is surveyed and used to establish a set of sensitivity estimates for these tests. These estimates will permit a comparison of the bioassays and will also give an estimate of the concentrations of toxic materials that could be in a mixture which registers negative in a particular test (i.e., What does a negative test response indicate?). However, evaluations such as this one can provide only the approximate sensitivity of the bioassays to chemical classes and the results are not applicable directly to the testing and evaluation of complex environmental samples.

Three tests, the Ames *Salmonella*/microsome mutagenesis assay, the *in vitro* rodent cell (CHO) clonal toxicity assay and the *in vivo* rodent toxicity assay all have substantial published data bases using study designs similar to those employed in Level 1 environmental assessment. The sensitivity limits for these three tests are summarized in this review. Methods developed to assess these data will be applicable to other Level 1 tests such as the rabbit alveolar macrophage (RAM) assay, aquatic tests and other ecological assays; however, these evaluations await the development of a sufficient data base on a wide variety of pure compounds. Some of the Level 1 bioassays (e.g., aquatic toxicity assays and RAM assay, etc.) have extensive data bases for responses with complex environmental samples but not with pure chemicals. The insect toxicity test (*Drosophila* LD_{50}) has a large base of published data but the test conditions are extremely variable, making an interpretation of the sensitivity difficult at the present time.

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I. INTRODUCTION

The primary objective of IERL/RTP Level I Environmental Assessment is to reliably evaluate stationary-source emissions for their toxic potential. With this information, emission sources and process streams can be ranked according to their estimated toxic potency and further testing can be applied in an efficient manner. The impact of control technology can also be evaluated from Level 1 test data.

Level 1 Environmental Assessment includes both chemical and biological testing of solid, liquid and gaseous effluent streams from stationary-site industry. The overall collection and analysis of the data use both sets of information as shown in Figure 1.

Properly performed and interpreted Level 1 assessments provide guidance for subsequent quantitative confirmation of the source's environmental impact or for application and evaluation of appropriate control technology.

The steps outlined in Figure 1 for Level 1 assessment do not constitute a quantitative risk assessment for particular emission sites. The bioassays used in this program are rapid and sensitive predictors of more complex toxicological processes and therefore are considered as detection tests rather than risk assessment tests.

One feature of Level 1 Environmental Assessment which needs to be addressed is the level of sensitivity for the chemical and biological methods. There is little problem in defining the limits of sensitivity in chemical analysis; however, the same type of definition for bioassays is extremely difficult. This is especially true for some of the bioassays because the data are both quantitative and qualitative. Table 1 outlines the proposed Level 1 bioassays and the classification of data derived from these tests. Qualitative responses are defined as either positive or negative, toxic signs or descriptions of growth. Quantitative effects are those which are specifically measured by counting colonies, animal deaths or growth-medium turbidity.

The purposes of this document are to discuss the problem of determining the levels of sensitivity in biological assays recommended for Level 1 Environmental Assessment as defined in the IERL/RTP Procedures Manual (1) and to summarize the sensitivity data where sufficient data exist. Data and sensitivity estimates have been developed for the Ames Salmonella/microsome mutagenesis assay, the rodent cell (CHO) clonal toxicity assay and the *in vivo* rodent acute toxicity assay.

The approach is oversimplified since it considers only the response of the Level 1 bioassays to pure compounds and assumes that responses are generally additive. Synergisms and antagonisms to biological response have been considered, but not addressed. Therefore, the results of an evaluation such as this one can provide only the range or approximate sensitivity of the assays surveyed to chemical classes; results are not applicable directly to the testing and evaluation of complex environmental samples.

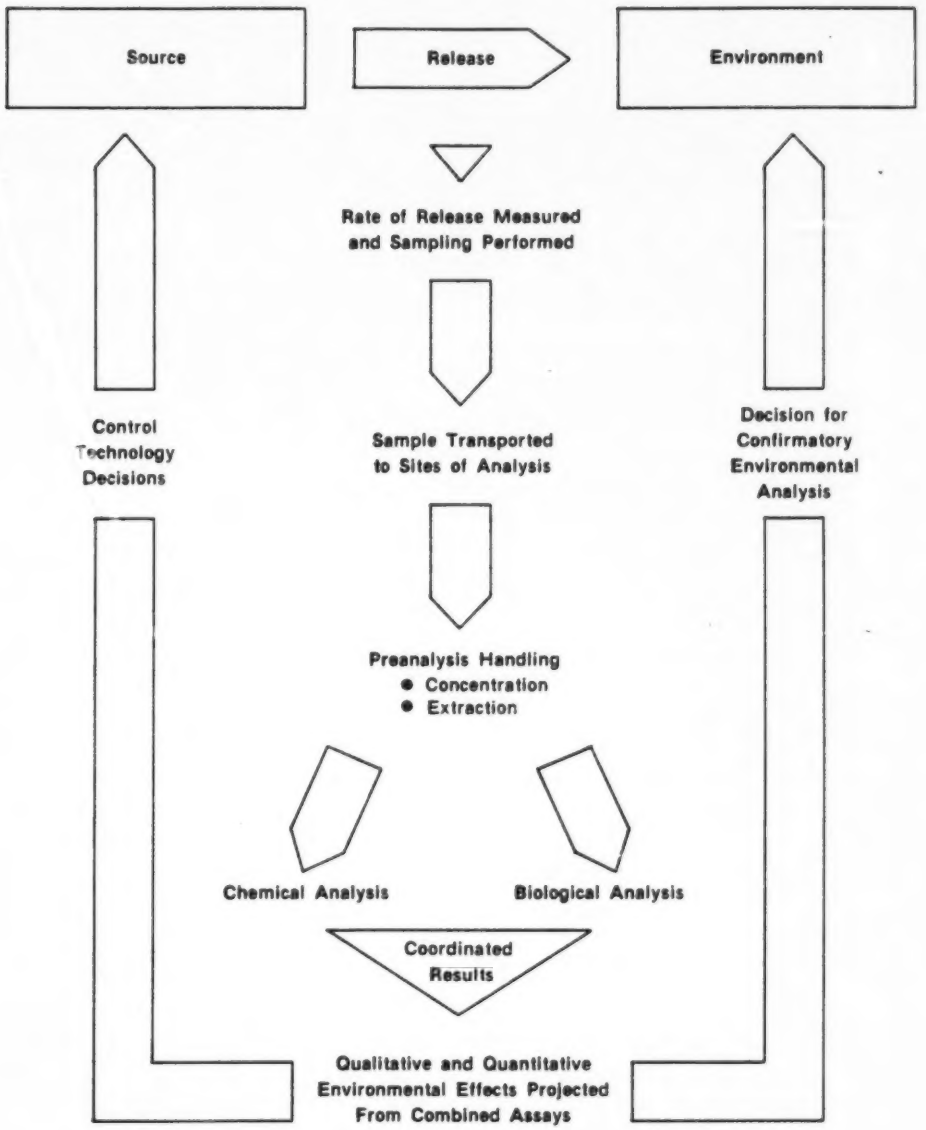


Figure 1 OVERVIEW OF TYPICAL STEPS INVOLVED IN LEVEL 1 ENVIRONMENTAL ASSESSMENT.

TABLE 1
TYPES OF DATA RESULTING FROM LEVEL 1 BIOASSAYS

Assay	Quantitative				Qualitative	
	Lethal Conc. (LC_{50})	Lethal Dose (LD_{50})	Effect. Conc. (EC_{50})	Min. Effect. Conc. (MEC)	Toxic Signs	Neg. or Pos.
HEALTH EFFECTS						
Ames Salmonella[a]				X		X
RAM Toxicity			X			
CHO Clonal Toxicity			X			
Rodent Toxicity		X			X	
AQUATIC ECOLOGICAL EFFECTS						
Freshwater Fish	X				X	
Freshwater Invertebrates			X			
Freshwater Algae[b]			X			X
Marine Fish	X				X	
Marine Invertebrates			X			
Marine Algae[b]			X			X
TERRESTRIAL ECOLOGICAL EFFECTS						
Plant Stress Ethylene			X		X	
Root Elongation			X			
Insect Toxicity[c]		X	X			X

[a] Ames data is evaluated as positive or negative (ND). Positive chemicals are further ranked as having low (L), moderate (M) or high (H) mutagenicity.
[b] Algal assay data may indicate inhibitory (negative) or stimulatory (positive) effects on growth.
[c] The insect toxicity test is evaluated using the LD_{50} while the optional insect fertility test is evaluated either as negative or positive or is ranked using the EC_{50}.

II. FACTORS INFLUENCING SENSITIVITY IN BIOLOGICAL ASSAYS

The proposed data formatting and evaluation scheme in "Level 1 Bioassay Assessment and Data Formatting" defines the concept of "no detectable toxicity" for each of the bioassays as no significant response at a maximum applied dose (2). This definition implies that the test sample may contain toxicants but they are below the degree of resolution inherent in the particular assay. The following list contains several factors that are important in influencing the resolution of a test:

1. The maximum dose/concentration that can be applied to the assay system (e.g., the maximum volume of material that can be gavaged into an adult male or female rat or the mg/plate dose reasonably applied to an Ames plate test).

2. The inherent response of the assay system to specific classes of chemicals (e.g., traces of heavy metals will produce toxicity in most mammalian cells at lower concentrations than halogenated organic compounds).

3. The number and site of critical targets in the test organism that must be affected to produce a lethal or toxicological response.

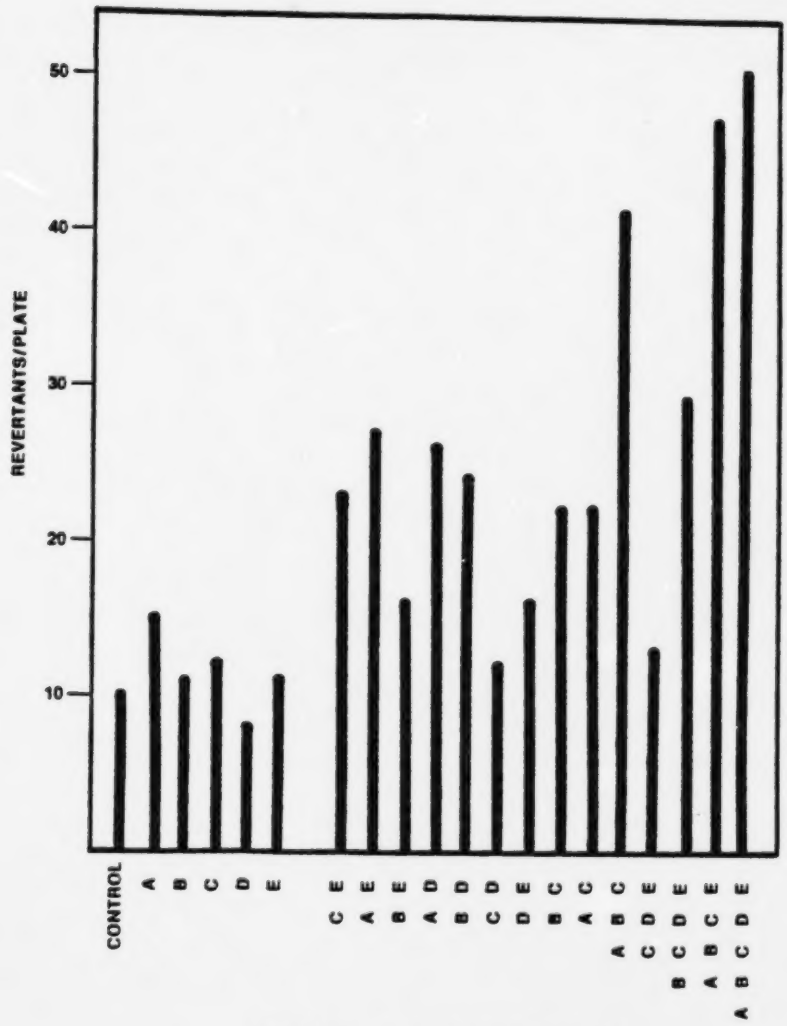
4. The validity of extrapolating dose response data for lethality and mutagenesis linearly down to low dose levels. In order to determine the minimum effective concentration (MEC), one must use data from the clearly detectable response range and extrapolate to a level showing a weak but significant effect (i.e., if the criterion for positive response in Salmonella strain TA-100 is a doubling over the background and if a chemical concentration of 100 µg/plate produced 1000 revertants/plate, the number of revertants/plate for a significant response with a background of 150 would be 300 and the MEC for that compound should be approximately 30 µg/plate).

5. The chemical interactions (synergistic and antagonistic) in complex mixtures. Some preliminary data shown in Figure 2 indicate that mutagens are synergistic if they act by a similar mechanism. This effect could considerably alter the MEC if calculated for single chemicals or classes of chemicals, but would provide a worse case in terms of synergistic effects (i.e., would increase likelihood of detection in bioassays).

6. The ability of the assay system to detoxify or eliminate the toxicant will impact on the limit of bioassay resolution and detectability.

7. The ability of the assay system to alter metabolically the toxicant to a more toxic or mutagenic form will affect the limit of bioassay resolution and detectability.

8. Nature of the test response. Tests with discontinuous data sets such as the Ames test are theoretically more amenable to this concept than are tests with continuous data sets. Likewise, the sensitivity of assays that produce only quantitative data can be interpreted with greater ease than assays that produce both quantitative and qualitative data, as summarized in Table 1.



STRAIN: S. typhimurium G-46

CHEMICAL	CODE LETTER	"NO EFFECT LEVEL/PLATE"
EMS	B	0.1% at 50 µl
MMS	C	0.1% at 50 µl
MNNG	A	0.5 µg/ml at 20 µl
TEM	E	1000 µl/ml at 50 µl
Na Azide	D	5 µl/ml at 10 µl

Individual compounds A-D at the concentrations selected cluster very near the spontaneous background of 10 revertants per plate. Multiple combinations [e.g., C + E, etc.] show, in most cases, synergistic effects, and to a certain degree, the interaction increases as the number of compounds increases. The importance of this effect is that it tends to increase the level of bioassay sensitivity by responding to groups of chemicals rather than individual compounds.

Figure 2 SYNERGISTIC ACTION WITH COMBINATIONS OF MUTAGENS

III. DATA REVIEW FOR SELECTED BIOASSAYS

A. Introduction

The following discussions center on the issue of Level 1 bioassay sensitivity to various chemicals. Published data for three Level 1 bioassays are reviewed and summarized. Difficulties encountered with this type of data compilation are discussed and recommendations made on how to provide a quantitative basis for the evaluation of the limits of Level 1 bioassay resolution and detectability.

Defining the lower limits of assay sensitivity is a difficult procedure. The critical factor in development of a data base of comparable information is protocol standardization. Data from bioassays using standardized procedures, such as the Ames Salmonella/microsome mutagenesis assay and the in vivo rodent acute toxicity assay, can be easily collected and compared. Problems arise when data from different protocols are compared. The published data from cell culture toxicity is of less value than the reported Ames or acute rodent toxicity data, since the concentration/exposure ratios vary considerably and few of the published studies are consistent with the Level 1 procedure which calls for a 24-hour exposure period.

The rather simplistic approach of comparing Level 1 data collected under similar conditions does not tell the entire story about the true level of assay sensitivity or relevance to environmental assessment. Considerations such as the slope of the response curve, the potential for bioaccumulation or biotransformation and the effect of chronic versus acute exposure ideally should not be ignored. In Figure 3, one can see two compounds with an identical LD_{50} (the dose of chemical that kills 50 percent of the test animals) but with toxicity curves of differing slopes. Compound A would be detected as toxic at much lower concentrations than compound B. Yet once compound B becomes toxic, it is highly lethal even at small increments over the LD_{50} value.

The interpretation of this exercise is based on the fact that, even though both compounds have identical LD_{50}'s, their modes of action and potential environmental effects are possibly quite different. While toxic effects of compound A can be detected at lower concentrations than compound B, the latter in fact may be the more hazardous since it rapidly becomes lethal above the LD_{50} dose in contrast with compound A. Conversely, A may be the more hazardous chemical at concentrations lower than the LD_{50} or if bioaccumulation occurs in the environment that concentrates compound A to toxic levels.

Additional factors such as the nature of the test response must be considered when evaluating Level 1 data for levels of sensitivity. The majority of Level 1 bioassays produce continuous data sets over the range of measured responses. A basic difference exists, however, between Ames mutagenicity data and toxicity data produced by the other assays. In the toxicity assays, any chemical may be expected to exhibit toxicity if tested at a high enough dose. Assay sensitivity is limited by the physical capacity of the system to accept test material and the length of time

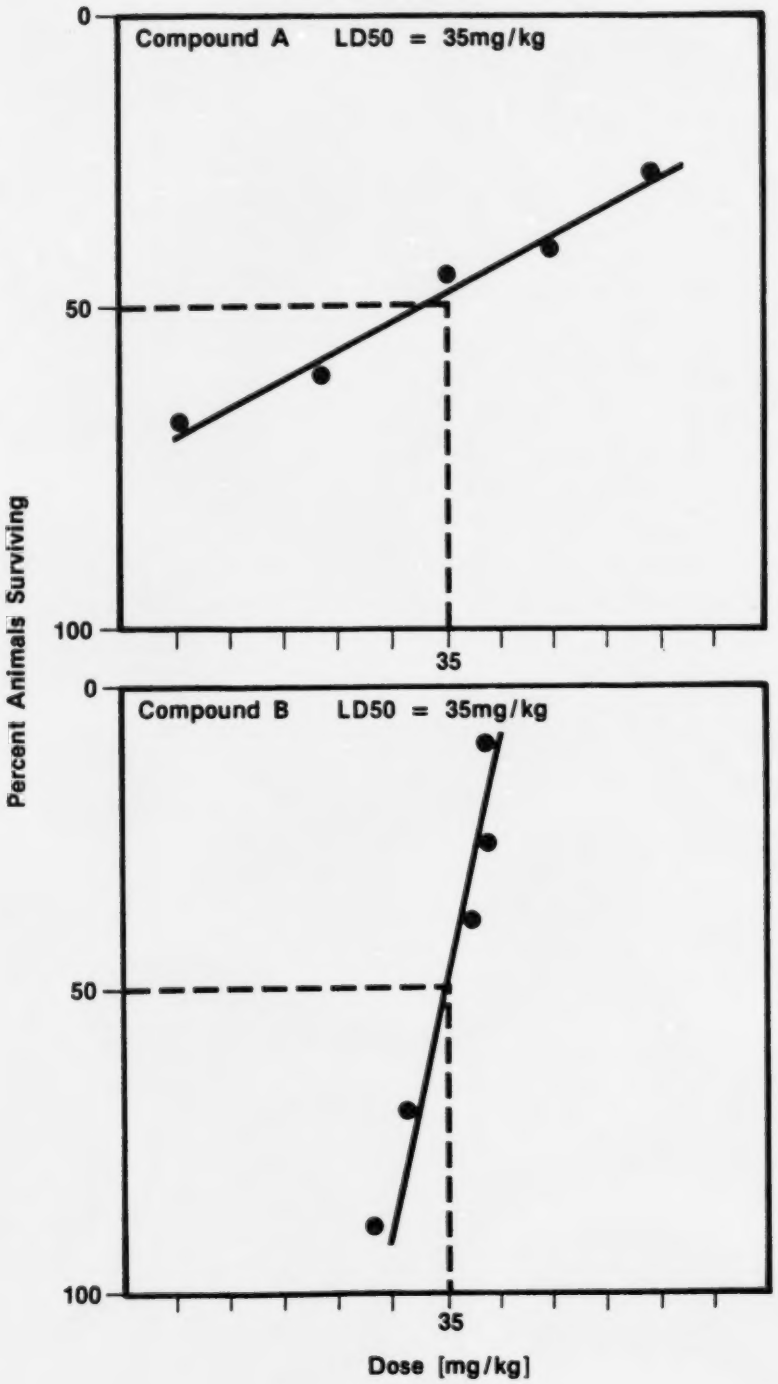


Figure 3 A COMPARISON OF DATA USED TO GENERATE LD50 VALUES.

during which the material is applied. In the Ames assay, on the other hand, a nonmutagenic compound will not produce a positive response regardless of applied dose. Ames data is therefore discontinuous (e.g., a chemical is either mutagenic or nonmutagenic) and only positive (mutagenic) chemicals exhibit a continuous data set. If a sample is positive, it can be categorized further as having high, moderate or low mutagenicity based upon the observed minimum effective concentration (Figure 4). A modicum of uncertainty exists in differentiating between nonmutagenic chemicals and weakly positive chemicals with activity below the threshold of Ames assay sensitivity. Chemicals in both situations are designated as having nondetectable mutagenicity.

Tests with clearly dichotomous responses (e.g., Ames test mutagenic or nonmutagenic) are more amenable to sensitivity evaluation than tests with continuous responses (e.g., EC_{50} in the CHO clonal toxicity test). For example, it appears that mutation induction is a "single hit" phenomenon and that the level of response is a function of total dose (concentration x exposure time). This means that one should obtain an equal response by elevating the concentration over a short exposure period or by extending the exposure period at a low concentration. Lethality, however, is generally not a "single hit" phenomenon, and distinctly different patterns of lethality will be obtained from experiments when the dose is held constant but the concentration and time parameters are varied. Thus, to define the lower limit effect as a function of dose is meaningful for the Ames test but not for bioassays measuring lethality unless the other parameters are specified. Examples of this will be shown in subsequent sections.

B. A Review of Ames Test Data

The Ames Salmonella/microsome mutagenicity assay has a well-accepted standardized protocol which has been adapted to Level 1 environmental assessment testing. There is a large amount of Ames mutagenicity data available that may be used to evaluate the levels of sensitivity and detectability of the Ames assay. For example, McCann et al. (3) have compiled a list of data from which this evaluation is drawn.

The minimum effective concentration (MEC expressed as amount-per-plate) can be calculated for various compounds and compared. For example, McCann et al. (3) list the mutagenic potency of 2-acetylaminoflourene as 108 revertant colonies/m mole or 4823 colonies per 10 µg using the tester strain TA-1538. This strain gives an average of 25 spontaneous revertant colonies per plate. If the criteria for a positive response is three times the spontaneous number of revertant colonies, the amount of 2-acetylaminofluorene per plate required to produce a positive response of 75 colonies is calculated to be 0.16 µg. This calculation relies on the observation that almost all of the hundreds of mutagens tested in Salmonella assays have shown linear dose-response which could be extended to extremely low dose ranges. However, there are a number of variables, such as the Salmonella strains, the amount of S-9 enzymes, the method of induction

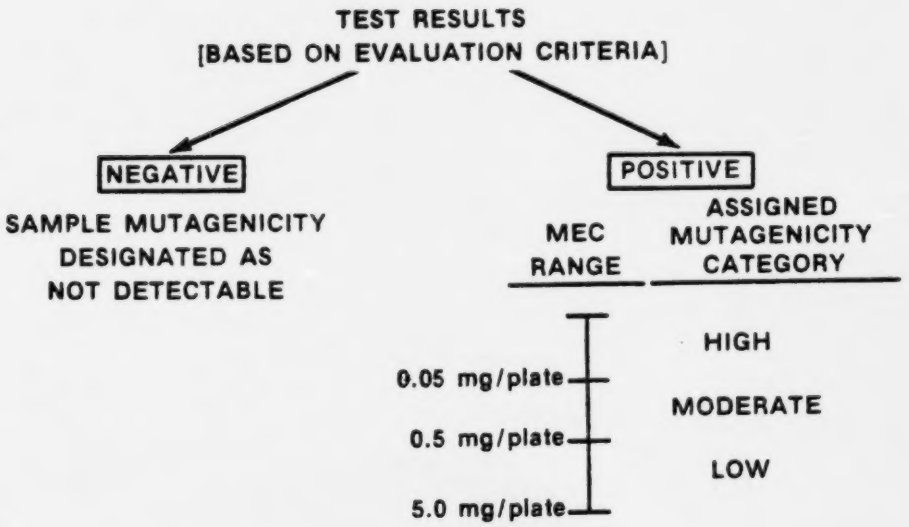


Figure 4 EVALUATION OF AMES TEST USING THE MAXIMUM APPLICABLE DOSE OF 5 mg PER PLATE

and the criteria for positive response, which can ultimately affect this minimum detectable concentration. In spite of these and other variables, this type of extrapolation can provide a simple method for approximating the minimum detectable concentrations of different classes of known mutagens in the Salmonella assay.

Table 2 summarizes the range of reported MEC values for different chemical classes. The chemicals reported in this section are organized by chemical family using the Multimedia Environmental Goals (MEG) (4) classification scheme. Table 3 includes MEC data for 90 chemicals also organized by chemical class. This compilation is by no means exhaustive and is not a representative selection of chemicals since most of the chemicals tested had known, or presumed, genetic activity.

In this manner, interpretation of response data can provide a valid basis by which the actual minimum detectable concentration can be compared with other data from standardized Level 1 Ames assays. The results of these experiments with pure chemicals can be used to establish the actual limit of sensitivity as well as the variability of this assay.

The level of sensitivity of the assay can be tested further by detecting mutagenic activity of various environmental samples into which:

(1) Minimum detectable concentration of a single known mutagen had been established (i.e., testing the hypothesis).

(2) A combination of different mutagens had been incorporated at a minimum detectable concentration level (Figure 2). This approach provides data on synergistic or antagonistic interaction of different combinations of mutagens in specific environmental samples.

The results summarized in Table 2 indicate a range of sensitivity from 3×10^{-3} to 4×10^{2} µg/plate for the Ames assay for the compounds tested. Based on a maximum applicable dose (MAD) of 5 mg/plate (or 200 µl/plate) in the test as currently run for Level 1 assessments, this sensitivity corresponds to 6×10^{-7} to 8×10^{-2} gram of chemical per gram of complex mixture for solid samples (or 1.5×10^{-5} to 2 grams of chemical per liter of aqueous sample). These response ranges may be compared to the corresponding chemical analyses if correction is made for the degree of concentration in the chemical analyses. In the solid sample case, the most sensitive Ames assay response (6×10^{-7} g/g) is on the order of the sensitivity of the corresponding least sensitive case chemical analysis (i.e. 10^{-6} to 10^{-7} g/g) (5). For aqueous liquid samples the Ames assay is less sensitive, yet it still compares well to Level 1 chemical analyses.

The data obtained from this type of analysis may provide valuable tools to establish the sensitivity limit of the Ames Salmonella assay in the detection of environmental mutagens. Existing data can be interpreted in terms of MEC and the sensitivity of the Ames Salmonella assay for compound classes can be postulated. However, the results of an evaluation such as this one can provide only the range or approximate sensitivity of this assay to chemical classes and are not applicable directly to the testing and evaluation of complex environmental samples.

TABLE 2

RANGE OF MINIMUM EFFECTIVE CONCENTRATION (MEC) VALUES IN THE LEVEL 1 AMES ASSAY SUMMARIZED BY CHEMICAL CLASS

MEG Group[a]	Chemical Class	No. of Entries	MEC[b] Range, Low and High Values (µg/plate)	Chemical
6	Glycols, Epoxides	4	7×10^{-2}	benzo (a) pyrene-4,5-oxide
			400	1,2,7,8-diepoxy-octane
8	Carboxylic Acids and Derivatives	6	3×10^{-3}	2-(2-furyl)-3-(nitro-2-furyl)-acrylamide (AF-2)
			64	melphalan
10	Amines	30	6×10^{-3}	2-aminofluorene
			210	N,N-dimethyl-4-(phenylazo) benzeneamine
14	Sulfonic Acid, Sulfoxides	2	49	methyl methane-sulfonate
			217	ethyl methane-sulfonate
16	Halogenated Aromatic Compounds	7	7.8×10^{-2}	9-10-dichloro-methyl anthra-cene
			2	10-bromoanthra-cene
17	Aromatic Nitro Compounds	6	1.4×10^{-2}	2-nitrosofluorene
			73.5	2-nitrosonaph-thalene
21, 22	Fused Polycyclic Hydrocarbons	17	1.3×10^{-1}	daunorubicin · HCl
			120	benzo(e)pyrene
23	Heterocyclic Nitrogen	9	1×10^{-2}	4-nitroquinoline-1-oxide
			38	uracil mustard

TABLE 2 (Continued)

RANGE OF MINIMUM EFFECTIVE CONCENTRATION (MEC) VALUES IN THE LEVEL 1 AMES ASSAY SUMMARIZED BY CHEMICAL CLASS

MEG Group[a]	Chemical Class	No. of Entries	MEC[b] Range, Low and High Values (µg/plate)	Chemical
24	Heterocyclic Oxygen Compounds	6	1.2×10^{-2}	aflatoxin B_1 and aflatoxicol
			42	aflatoxin B_2
25	Heterocyclic S Compounds	1	11	hycanthone methanesulfonate
26	Organophosphorus Compounds	2	1.1	cyclophosphamide
			60	isophosphamide

[a]MEG = Multimedia Environmental Goals (4); chemical classification scheme developed as part of EPA Level 1 environmental assessment testing program. Some chemicals may be placed in more than one group.

[b]MEC = Minimum Effective Concentration. The minimum amount of test material required to give a positive response in the most sensitive tester strain.

TABLE 3

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
6	GLYCOLS, EPOXIDES				
	Benzo (a) pyrene-4,5-oxide	7	0.0014	TA1538	-
	1,2,3,4-diepoxybutane	4200	0.84	TA1535	-
	1,2,7,8-diepoxyoctane	40000	8.0	TA100	-
	2,-3-epoxypropional-dehyde (glycidaldehyde)	100	0.02	TA100	-
8	CARBOXYLIC ACIDS AND DERIVATIVES (AMIDES, ESTERS)				
	N,N-bis(2-chlorethyl)-2-naphthylamide	1360	0.27	TA100	A
	N-hydroxy-N-(2-fluorenyl) acetamide	40	0.008	TA98	A
	Melphalan	6400	1.28	TA1535	A
	2-oxetanone (β-propiolactone)	500	0.1	TA100	-
	2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide [AF-2]	0.3	0.00006	TA100	-
	N-(trichloromethyl(thio-4-cyclo-hexene 1,2-dicarboxyimide (captan)	340	0.068	TA100	-
10	AMINES				
	2,4-diaminotoluene	2800	0.56	TA1538	A
	4-aminobiphenyl	150	0.03	TA100	A
	4,4'-diaminobiphenyl (benzidine)	1400	0.28	TA1538	P
	2',3-dimethyl-4-aminobiphenyl	86	0.017	TA100	A

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
	AMINES (Continued)				
	4,4'-methylene-bis 2-chloroaniline [MOCA]	2660	0.53	TA100	A
	N,N-dimethyl-4-(phenylazo) benzenamine	21000	4.2	TA98	A
	2-methyl-4-dimethylaminoazo-benzene	3200	0.64	TA98	A
	N,N-dimethyl-4- (3-methylphenyl) azo benzenamine	5300	1.06	TA1538	A
	3-methoxy-4-aminoazobenzene	2.3	0.00046	TA1538	A
	2-methyl-4- (2-methylphenyl)azo benzenamine	4200	0.84	TA100	A
	4-amino-trans-stillbene	90	0.018	TA1538	P
	4-dimethylamino-trans-stilbene	90	0.018	TA1538	P
	1-naphthylamine	9600	1.92	TA100	A
	2-naphthylamine	70	0.014	TA100	A
	N-(1-naphthyl)hydroxylamine	20	0.004	TA100	-
	N-(2-naphthyl)hydroxylamine	2100	0.42	TA100	-
	1-aminoanthracene	243	0.049	TA98	P
	2-aminoanthracene	3	0.0006	TA98	P
	2-aminofluorene	0.6	0.00012	TA98	P
	N-hydroxy-2-aminofluorene	2.5	0.0005	TA98(80)	-

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
	AMINES (Continued)				
	2-acetylaminofluorene	16	0.0032	TA1538	P
	N-acetoxy-2-acetylaminofluorene	50	0.01	TA98	-
	2,7-diaminofluorene	250	0.05	TA98	A
	2,7-bis-acetylaminofluorene	222	0.044	TA1538	P
	6-aminochrysene	12	0.0024	TA1538	A
	4-hydroxyaminoquinoline-1-oxide	60	0.012	TA100	-
	3,6-bis-dimethylaminoacridine	120	0.024	TA100	A
	aziridine	130	0.026	TA1535	-
	2-methylaziridine	170	0.034	TA1535	-
	nitrogen mustard	900	0.18	TA1535	-
14	SULFONIC ACIDS, SULFOXIDES				
	methyl methanesulfonate (MMS)	4900	0.98	TA100	-
	ethyl methanesulfonate (EMS)	21700	4.34	TA100	-
16	HALOGENATED AROMATIC COMPOUNDS				
	10-bromomethylanthracene	200	0.04	TA100	-
	7-bromomethyl-12-methylbenz(a) anthracene	130	0.026	TA98	-
	7-chloromethylbenz(a)anthracene	60	0.012	TA100	-
	10-chloromethyl-9-chloro-anthracene	140	0.028	TA100	-

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
	HALOGENATED AROMATIC COMPOUNDS (Continued)				
	10-chloromethyl-9-methyl-anthracene	130	0.026	TA1537	-
	7-chloromethyl-12-methyl-benz(a)anthracene	100	0.02	TA100	-
	9,10-dichloromethyl anthracene	7.8	0.0016	TA1537	-
17	AROMATIC NITRO COMPOUNDS				
	4-nitrobiphenyl	525	0.11	TA100	-
	2-nitronaphthalene	525	0.11	TA100	-
	5-nitroacenaphthene	310	0.062	TA100	A
	2-nitrofluorene	310	0.062	TA1538	-
	2-nitrosonaphthalene	7350	1.47	TA1538	-
	2-nitrosofluorene	1.4	0.00028	TA1538	-
21,22	FUSED POLYCYCLIC HYDROCARBONS				
	Benz(a)anthracene	600	0.12	TA100	A
	7-methylbenz(a)anthracene	350	0.07	TA100	A
	7,12-dimethylbenz(a)anthracene	380	0.076	TA100	A
	12-methylbenz(a)anthracene-7-methanol	250	0.05	TA100	-
	Benz(a)phenanthrene(chrysene)	17	0.0034	TA100	A
	15,16-dihydro-11-methyl-cyclopenta(a)phenathrene-17-one	83	0.017	TA100	A

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
	FUSED POLYCYCLIC HYDROCARBONS (Continued)				
	daunorubicin · HCl	13	0.0026	TA98	-
	adriamycin · HCl	43	0.0086	TA98	-
	benzo(a)pyrene	59	0.012	TA100	A
	3-hydroxybenzo(a)pyrene	19	0.0038	TA100	A
	6-hydroxymethylbenzo(a)pyrene	30	0.006	TA100	A
	7,8-dihydrobenzo(a)pyrene	94	0.019	TA1538	A
	benzo(e)pyrene	12000	2.4	TA100	A
	3-methylcholanthrene	130	0.026	TA100	A
	dibenz(a,c)anthracene	46	0.0092	TA100	A
	dibenz(a,h)anthracene	700	0.14	TA100	A
	dibenzo(a,i)pyrene	420	0.084	TA100	A
23	HETEROCYCLIC NITROGEN COMPOUNDS				
	7,9-dimethylbenz(c)acridine	770	0.154	TA100	A
	7,10-dimethylbenz(c)acridine	490	0.098	TA100	A
	dibenz(a,j)acridine	440	0.088	TA100	A
	1,2-dimethyl-5-nitroimidazole	98	0.02	TA100	-
	1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole	4200	0.84	TA100	-
	ICR-10	150	0.03	TA1537	-

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
	HETEROCYCLIC NITROGEN COMPOUNDS (Continued)				
	ICR-170	3.9	0.00078	TA1537	-
	4-nitroquinoline-1-oxide	1.8	0.00036	TA100	-
	uracil mustard	3800	0.76	TA1535	-
24	HETEROCYCLIC OXYGEN COMPOUNDS				
	aflatoxin B_1	1.2	0.00024	TA100	A
	aflatoxin B_2	4200	0.84	TA100	A
	aflatoxin G_1	23	0.0046	TA98	P
	aflatoxin M_1	23	0.0046	TA98	P
	aflatoxicol	1.2	0.00024	TA98	P
	sterigmatocystin	9.9	0.002	TA100	A
25	HETEROCYCLIC S COMPOUNDS				
	hycanthone methanesulfonate	1100	0.22	TA1538	-

TABLE 3 (Continued)

MINIMUM EFFECTIVE CONCENTRATION (MEC) OF MUTAGENS
FOR POSITIVE RESPONSE IN THE AMES ASSAY

MEG[a] Group	Chemical	MEC[b] µg/plate (x 10^{-2})	% of MAD[c] To Observe MEC[b]	Strain	S-9[d]
26	ORGANOPHOSPHROUS COMPOUNDS				
	Cyclophosphamide	110	0.022	TA1535	A
	Isophosphamide	6000	1.2	TA1535	A

[a]MEG = Multimedia Environmental Goals (4); chemical classification scheme developed as part of EPA Level 1 environmental assessment testing program. Some chemicals may be placed in more than one group.

[b]MEC = Minimum Effective Concentration. Minimum amount of mutagen per plate calculated from data of McCann et al. (3) to give a positive mutagenic response. The criteria for a positive response are three times the number of spontaneous colonies for TA1535, TA1537 and TA1538 and two times the number of spontaneous revertant colonies for TA98 and TA100. The numbers of revertant colonies required for a positive response in each strain were as follows: TA1535, 60; TA1537, 21; TA1538, 75; TA98, 80; and TA100, 280.

[c]MAD = Maximum Applicable Dose. The recommended maximum amount of test material per plate that can be tested. The MAD for the Ames Assay is 5000 µg/plate.

[d]S-9 fraction from rat liver induced with either Aroclor 1254 (A) or phenobarbitol (P). (-) indicates S-9 fraction was not added to the assay.

C. A Review of Rodent Toxicity Data

Level 1 acute rodent toxicity testing is a valuable test method for toxicological assessment of complex effluents. The advantages of the in vivo toxicity assays lie mainly in the fact that the testing is performed in whole animals. Also there is a significant background of rodent test data on a wide range of toxicants using standard test protocols, thus supplying needed information for analysis of levels of assay sensitivity and for reliable interpretation of results with complex effluents. The primary disadvantage of the assay is the inability to predict the toxicity induced by long-term/low-level exposures.

This section presents acute oral LD_{50} values for a series of chemicals tested in mice using standard test protocols. These protocols are similar to the rodent toxicity assay described in the procedure manual for Level 1 environmental assessment biological tests (1). The chemicals reported in the section are organized by chemical family using the Multimedia Environmental Goals (MEG) classification scheme (4). These MEG categories were devised as part of the EPA Level 1 environmental assessment testing program. A total of 98 chemicals is reported representing 20 organic chemical groups as well as inorganic and metallic compounds.

The method developed for this assessment uses the published acute oral LD_{50} values for mice. If one assumes that a pure chemical (100 percent) is given orally, then it should be detected at the MAD if its LD_{50} is $\leq$ 10 g/kg (10,000 mg/kg). Therefore, one can assume for a pure chemical that 100 percent is equal to 10,000 mg/kg. Given a chemical which has an LD_{50} of 100 mg/kg, an oral dose of pure substance administered at the MAD would probably kill all the animals. The same chemical, if present at a 50 percent concentration, given at the MAD would still be very toxic (an effective dose of 50 times the LD_{50}). At a concentration of 10 percent in the sample, this compound would still be detected easily, as the effective dose would be 10 times the LD_{50}. Not until one reaches a concentration of less than 1 percent (an effective dose of less than the LD_{50} when administered at the MAD) would the likelihood of failure to detect this substance in the rodent toxicity assay be significant. Thus, based on the assumption of dose response linearity, the limit of detectability of the model compound would be near a 1 percent concentration in a test sample which is administered at the MAD.

Table 4 presents a summary of the range of LD_{50} values encountered for each chemical (MEG) category in the review of rodent toxicity data. Table 5 provides the complete listing of reviewed chemicals broken down into MEG categories. The dose required to kill 50 percent of test animals (LD_{50}) is reported as milligrams of chemical per kilogram of animal body weight. Using the approach discussed earlier, the sensitivity of the assay to each chemical is determined. A convenient method of analysis is to report the minimum concentration (as a percentage of the maximum applicable dose) required for a chemical in a mixture for the LD_{50} to be observed for that chemical. This assumes that only the chemical in question exerts toxicity and that no antagonistic or synergistic effects

occur. Analysis of such hypothetical mixtures should be considered with caution and not applied directly to the evaluation of complex environmental mixtures.

Several other assumptions are required in analyzing these data: 1) all LD_{50} curves are roughly similar; 2) they extrapolate to zero dose; 3) based on the LD_{50} value of the pure chemical, at least one animal of either sex would die in the Level 1 Quantal assay (preliminary screening at the MAD) if a complex mixture contained the stated concentrations of the chemical.

Toxicity data in Table 5 are evaluated in two ways based on the age of the test animal. The current Level 1 rodent toxicity test is performed using weanling mice (21-28 days old) while earlier protocols recommended young adult mice. The change in animal age (and physical size) necessitated a reduction in the MAD from 10 g/kg for young adult mice to 5 g/kg for weanling mice. The minimum concentration of a chemical (reported as a percentage of the MAD) in a mixture required to observe the LD_{50} is based on both the 10 g/kg and 5 g/kg MAD levels. Therefore, 100 percent of the MAD for weanlings would be 5 g/kg while the corresponding level for young adults would be 10 g/kg.

Several observations can be made from Tables 4 and 5. Only about one-third of the chemicals listed would be expected to kill at least one animal in the Quantal assay when present at 1 percent or less (with young adult mice: 10 g/kg MAD). It is probably rare that individual pure chemicals reach this level in most environmental samples. Based on the number of chemicals which must reach a concentration of $\geq$ 5 percent of the total sample ($\geq$ 10 percent for weanlings), the comparative insensitivity of the Quantal test is not surprising. This survey was not exhaustive, but it emphasizes the limited nature of published acute oral LD_{50} values for pure chemicals. LD_{50} values by all other routes of administration were omitted from this survey. Caution should be used in relating levels of sensitivity of this assay to environmental samples because this review of pure chemicals provides little insight into the toxicity of complex mixtures.

TABLE 4

RANGE OF LD_{50}[a] VALUES IN THE LEVEL 1 RODENT TOXICITY ASSAY SUMMARIZED BY CHEMICAL CLASS

MEG Group[b]	Chemical Class	No. of Entries	LD_{50} Range, Low and High (g/kg)	Chemical
1	Aliphatic hydrocarbons	2	800	1-nitropropane
			1440	nitromethane
2	Alkyl halides	7	81	2-chloroethanol
			3160	trichlorovinyl-silicone
3	Ethers	1	170	bis(2,3-epoxy-propyl) ether
6	Glycols, Epoxides	2	238	9-epichlorohydrin
			4200	2-ethyl-1,3-hexanediol
7	Aldehydes, Ketones	5	21	acrolein
			1300	cyclohexanone
8	Carboxylic Acids and Derivatives	9	52	2-fluoroacetamide
			5040	citric acid
9	Nitriles	2	65	phthalonitrile
			78	phenylaceto-nitrile
10	Amines	9	20	2,2-dichloro-N-methyl-diethyl-amine HCL
			1625	2,4-diamino-toluene
11	Azo Compounds; Hydrazine Derivatives	5	33	1-methylhydrazine
			265	1,1-dimethyl-hydrazine
12	Nitrosamines	1	3850	N-nitrosodiphenyl-amine

TABLE 4 (Continued)

RANGE OF LD_{50}[a] VALUES IN THE LEVEL 1 RODENT TOXICITY ASSAY SUMMARIZED BY CHEMICAL CLASS

MEG Group[b]	Chemical Class	No. of Entries	LD_{50} Range, Low and High (g/kg)	Chemical
13	Thiols, Sulfides and Disulfides	2	38	methylsulfide
			3700	tris (1-aziridinyl) phosphine sulfide
14	Sulfonic Acids, Sulfoxides	2	2000	sodium lauryl sulfonate
			2100	alkyl aryl sulfonate
15	Benzene; Substituted Benzene Hydrocarbons	8	16	n-cumenol methyl carbamate
			3900	anthranilic acid methylester
16	Halogenated Aromatics	7	50	aldrin
			4000	hexachlorobenzene
17	Aromatic Nitro Compounds	3	135	1-chloro-2-nitrobenzene
			812	p-nitroaniline
18	Phenols	4	300	phenol
			1600	propylgallate
20	Nitrophenols	1	45	2,4-dinitrophenol
21,22	Fused Polycyclic Hydrocarbons	2	438	carbonyl
			700	phenanthrene
23	Heterocyclic Nitrogen Compounds	4	125	diquat
			3340	nicotine
26	Organophosphorus Compounds	2	135	dichlorvos (DDVP)
			292	metepa

TABLE 4 (Continued)

RANGE OF LD_{50}[a] VALUES IN THE LEVEL 1
RODENT TOXICITY ASSAY SUMMARIZED
BY CHEMICAL CLASS

MEG Group[b]	Chemical Class	No. of Entries	LD_{50} Range, Low and High (g/kg)	Chemical
	Metals and Organometallic Compounds[c]	18	4	sodium dimethyl-arsinate
			4000	sodium chloride

[a] LD_{50} = Dose lethal to 50 percent of animals.

[b] MEG = Multimedia Environmental Goals classification scheme (4).

[c] Metals and organometallic compounds are summarized as a group and not as individual elements.

TABLE 5

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c] Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
1	ALIPHATIC HYDROCARBONS			
	1-nitropropane	800	8.0	16.0
	nitromethane	1440	14.4	28.8
2	ALKYL HALIDES			
	2-chloroethanol	81	0.8	1.6
	1-chloro-1-nitro-propane	510	5.1	10.2
	1,2-dichloroethane	600	6.0	12.0
	1,2-dichloropropane	510	5.1	10.2
	hexachlorobutadiene	110	1.1	2.2
	hexachlorobutane	2000	20.0	40.0
	trichlorovinyl silicone	3160	31.6	63.2
3	ETHERS			
	bis(2,3-epoxypropyl) ether	170	1.7	3.4
6	GLYCOLS, EPOXIDES			
	9-epichlorohydrin	238	2.4	4.8
	2-ethyl-1,3-hexanediol	4200	42.0	84.0
7	ALDEHYDES, KETONES			
	acrolein	40	0.4	0.8

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c] Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
	ALDEHYDES, KETONES (Continued)			
	benzaldehyde	28	0.3	0.6
	chloral hydrate	1100	11.0	22.0
	chloroacetaldehyde	21	0.2	0.4
	cyclohexanone	1300	13.0	26.0
8	CARBOXYLIC ACIDS AND DERIVATIVES (AMIDES, ESTERS)			
	acrylamide	170	1.7	3.4
	adipic acid	1900	19.0	38.0
	allyl formate	136	1.4	2.7
	bromoacetic acid	100	1.0	2.0
	citric acid	5040	50.4	(100.8)
	2-fluoroacetamide	52	0.5	1.0
	fusidic acid	1200	12.0	24.0
	iodocacetic acid	83	0.8	1.7
	salicylamide	1400	14.0	28.0
9	NITRILES			
	phenylacetonitrile	78	0.8	1.6
	phthalonitrile	65	0.7	1.3

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c]	
			Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
10	AMINES			
	allylamine	57	0.6	1.1
	benzidine	214	2.1	4.3
	1,3-cyclohexanediamine	543	5.4	10.9
	2,4-diaminotoluene	1625	16.3	32.5
	2,2-dichloro-N-methyl diethylamine HCl	20	0.2	0.4
	diethylamine	649	6.5	13.0
	emetine dihydrochloride	44	0.4	0.9
	hydroxylamine, HCl	400	4.0	8.0
	triethylamine	546	5.5	10.9
11	AZO COMPOUNDS, HYDRIAZINE DERIVATIVES			
	1,1-dimethylhydrazine	265	2.7	5.3
	hydrazine	59	0.6	1.2
	1-methylhydrazine	33	0.3	0.7
	semicarbazide	176	1.8	3.5
	thiosemicarbazone benzaldehyde	100	1.0	2.0
12	NITROSAMINES			
	N-nitrosodiphenylamine	3850	38.5	77.0

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c] Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
13	THIOLS, SULFIDES AND DISULFIDES			
	methyl sulfide	3700	37.0	74.0
	tris (1-aziridinyl) phosphine sulfide	38	0.4	0.8
14	SULFONIC ACIDS, SULFOXIDES			
	alkyl aryl sulfonate	2010	20.1	40.2
	sodium lauryl sulfonate	2000	20.0	40.0
15	BENZENE; SUBSTITUTED BENZENE HYDROCARBONS			
	anthranilic acid, methyl ester	3900	39.0	78.0
	benzoic acid	2370	23.7	47.4
	benzyl alcohol	1580	15.8	31.6
	m-cumenol methyl carbamate	16	0.2	0.3
	methyl styrene	3160	31.6	63.2
	salicylic acid acetate	815	8.2	16.3
	styrene	316	3.2	6.3
	p-tert-butyl toluene	900	9.0	18.0
16	HALOGENATED AROMATIC COMPOUNDS			
	aldrin	50	0.5	1.0
	p-chloroaniline	100	1.0	2.0

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c] Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
	HALOGENATED AROMATIC COMPOUNDS (Continued)			
	α-chloronapthalene	1091	10.9	21.8
	DDT	135	1.4	2.7
	dieldrin	114	1.1	2.3
	hexachlorobenzene	4000	40.0	80.0
	1,2,4,5-tetrachloro-benzene	1035	10.4	20.7
17	AROMATIC NITRO COMPOUNDS			
	1-chloro-2-nitro-benzene	135	1.4	2.7
	o-nitroaniline	308	3.1	6.2
	p-nitroaniline	812	8.1	16.2
18	PHENOLS			
	cresol	816	8.2	16.3
	hydroquinone	400	4.0	8.0
	phenol	300	3.0	6.0
	propyl gallate	1600	16.0	32.0
20	NITROPHENOLIC COMPOUNDS			
	2,4-dinitrophenol	45	0.5	0.9

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c] Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
21,22	FUSED POLYCYCLIC HYDROCARBONS			
	carbaryl	438	4.4	8.8
	phenanthrene	700	7.0	14.0
23	HETEROCYCLIC NITROGEN COMPOUNDS			
	benzimidazole	2910	29.1	58.2
	1-carbethoxy-1,2-dehydroquinoline	160	1.6	3.2
	diquat	125	1.3	2.5
	nicotine	3340	33.4	66.8
26	ORGANOPHOSPHORUS COMPOUNDS			
	dichlorvos (DDVP)	135	1.4	2.7
	metepa	292	2.9	5.8
	METALS; ORGANOMETALLIC COMPOUNDS[e]			
38	aluminum chloride	3800	38.0	76.0
49	arsenic acid, Mg++	315	3.2	6.3
49	arsenic trioxide	45	0.5	0.9
32	beryllium fluoride	100	1.0	2.0
37	boric acid	3454	34.5	69.1
74	cobalt chloride	80	0.8	1.6
74	cobalt nitroprusside	74	0.7	1.5

TABLE 5 (Continued)

COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 RODENT TOXICITY BIOASSAY[a]

MEG Category[b]	Chemical	Acute Oral Mouse LD_{50}[c] (mg/kg)	PERCENT OF MAD[d] REQUIRED TO OBSERVE LD_{50}[c]	
			Adult (MAD[d] 10g/kg)	Weanling (MAD 5g/kg)
	METALS; ORGANOMETALLIC COMPOUNDS (Continued)			
79	colloidal silver	100	1.0	2.0
37	decaborane	40	0.4	0.8
72	ferrous sulfate, heptahydrate	1520	15.2	30.4
83	mecurous chloride	10	0.1	0.2
83	mecuric cyanide	33	0.3	0.7
54	selenium sulfide	370	3.7	7.4
28	sodium azide	27	0.3	0.5
28	sodium chloride	4000	40.0	80.0
49	sodium dimethylarsinate	4	0.04	0.08
41	thallium (I) sulfate	29	0.3	0.6
81	zinc chloride	350	3.5	7.0

[a]Data obtained from references 6 and 7.

[b]MEG = Multimedia Environmental Goals classification scheme (4).

[c]LD_{50} = Dose lethal to 50 percent of the animals.

[d]MAD = Maximum Applicable Dose. The recommended maximum amount of test material that can be tested.

[e]Metals and organometallic compounds are reported as a group and not as individual elements.

D. A Review of In Vitro Mammalian Clonal Toxicity Assays

1. Introduction

Mammalian *in vitro* clonal toxicity assays provide a sensitive and reliable method to measure and compare the cytotoxicity of test agents. The Chinese hamster ovary cell clonal toxicity assay is routinely used to measure the toxicity of environmental samples submitted under EPA Level 1 testing of point-source emissions. The measured end point is the inhibition of colony formation as a function of dose. The standard parameter for comparison is the dose necessary to reduce the colony-forming ability of quantitatively plated mammalian cells by 50% (the EC50 value). These survival data are continuous over the doses tested and the EC50 may be determined statistically or graphically from the data.

Quantitative comparisons of toxicity data can be made only from assays conducted under comparable conditions. A review of the literature must be selective in only grouping toxicity data collected under a standard range of conditions. This section will briefly review the theoretical and practical bases of clonal toxicity measurement. Criteria will be developed for grouping cytotoxicity data for comparison. Finally there will be a review of selected *in vitro* mammalian cytotoxicity data.

The addition of mammalian *in vitro* cytotoxicity assays to the array of Level 1 bioassays has several important consequences. The evolutionary distance between bacteria (*Salmonella*) and mammalian cells creates different biochemical and structural features which will bias the level of response to chemicals. Mammalian cells (eukaryotes) differ from bacteria (prokaryotes) in their level of organization and repair of DNA, mechanisms of metabolism of chemicals, organization of cellular membranes and other related cytologic structures and functions. Since the measured end point of cell killing (actually, reproductive death) is nonspecific, it can result from any number of different biological alterations such as disruption of enzyme systems, mutations, gross chromosome alterations, rupture of cell membranes, etc. Mammalian cells, therefore, may provide a different and relevant measure of cytotoxicity for health effects evaluation of Level 1 environmental samples.

In some cases the general toxicity will be similar between mammalian cells and prokaryotes and in others there will be a divergence. The chemical sodium azide is an example where the toxicities are parallel (8). Examples of environmental agents showing a distinct divergence in response are metals and physical agents such as asbestos. It has been shown that metal cations (salts of beryllium, cadmium, cobalt, manganese and nickel) were both cytotoxic and mutagenic in Chinese hamster V79 cells (9,10). This type of sensitivity is not observed in bacteria.

Of potentially greater importance to the Level 1 array of bioassays was the observation by Huang (11) that amosite, chrysotile or crocidolite asbestos were phagocytized (engulfed) by Chinese hamster ovary (CHO)

cells. Huang also reported that asbestos increased the frequency of HGPRT mutants in CHO cells. This report, therefore, demonstrates not only that mammalian cells are capable of responding in a sensitive manner to agents not toxic or mutagenic in bacteria but that the CHO target cells are capable of phagocytizing toxic particulate matter. This observation is supported by the study by Garrett (12) which indicated comparable phagocytic rates of model particulates and fly ash particles as well as particulate effluent from coal gasification, fluidized bed combustion and conventional coal combustion processes by both CHO cells and rabbit alveolar macrophages (RAM) cells. Slightly enhanced initial rates of phagocytosis were attributed to the mobility of RAM cells. These observations tends to support the recommendations to expand the use of the CHO clonal toxicity assay in Level 1 assessments. More useful information pertaining to potential health effects may be gained by expanding the endpoint evaluation in CHO cells to include genetic effects such as mutations, chromosome alterations or sister chromatid exchange (SCE). Especially since cell toxicity and mutation induction are independent functions, one cannot always be predicted from the other (13).

2. Criteria for Clonal Toxicity Evaluation

Much of the current testing technology in environmental and genetic toxicology results from the ability to study mammalian cells quantitatively in the single-cell state. Development of techniques to plate single mammalian cells onto a solid substrate and to have them grow into discrete macroscopic colonies (analogous to bacterial colonies on an agar-containing media), permits several basic operations. First, the fraction of viable cells capable of growth into visible colonies in a given population can be determined. Second, dose-response curves can be constructed, which measure the number of cells surviving exposure to given doses of a test agent. Third, the specific molecular requirements or sensitivities of single cells can be determined. Finally, the most important advantage of single-cell plating is that it permits isolated single cells to reproduce to form colonies of genetically uniform cells.

The Level 1 CHO clonal toxicity test uses the advantages of single cell plating to measure the reduction in colony-forming potential of plated CHO cells. The average number of surviving colonies at each dose level is compared to an appropriate control to give the percentage of survival. Survival curves are then constructed which graphically represent the measured cytotoxicity as a function of dose. The dimensions for graphic presentation are generally percentage of survival versus concentration (usually on a weight per volume, volume per volume or molar concentration basis).

Evaluation of cytotoxicity data in the numerical, and especially in the graphic, form permits rapid measurement of standard toxicity parameters. Toxicity data interpretation readily provides EC_{50} values, quantitative and relative ranking of the toxicity of various test agents and a subjective insight into the nature of the toxic action as reflected by the slope of the linear phase of the dose-response curve. Other cytotoxicity parameters which are of less importance are the mean lethal dose (the dose needed to reduce a population by 63 percent) and the hit number

(the y- axis intercept of the linear portion of the toxicity curve). The most convenient measure of cytotoxicity is the EC_{50} value which provides a biologically meaningful index for chemical toxicity evaluation, comparison and ranking.

The major variable in reported cytotoxicity data is in the time during which the test agent is exposed to the indicator cells. The applied dose is a function of both test-agent concentration and of the time of exposure. In this sense, the effective dose of a test material theoretically may be doubled by doubling the concentration, the exposure time, or by any intermediate combination of increased concentration and time which will equal a doubling of dose. Practically, this relation of concentration and time often does not hold true because of the complex nature of environmental samples and the many different mechanisms by which cells may be killed. Because of the composite nature of dose, and the often non-linear relation between concentration and time, quantitative comparisons of sensitivity and cytotoxicity data between assays conducted under different conditions become meaningless if toxicity data are discussed in terms of concentration alone. This underlines the importance of a unified testing protocol which would standardize the body of data reported. Most cytotoxicity assays alter concentration over a fixed time in order to vary dose. Notable exceptions would arise when the physical characteristics of the test agent preclude altering concentration such as with fixed-strength radiation sources and gaseous or volatile substances.

Mammalian in vitro clonal toxicity data in the literature have tended to be supportive work for a more complicated test than for toxicity as an end unto itself. Quite often, reported toxicity data are range-finding studies for subsequent assays to detect mutation, transformation, chromosomal alteration or unscheduled DNA synthesis. Exposure times for toxicity data therefore reflect treatment times called for in the more sophisticated test protocols. As a result, the available body of reported toxicity data varies greatly in exposure time, as well as in indicator cell type, attachment time, cell density and media.

A standard exposure period of between 16 and 24 hours has been selected to compare the sensitivity of clonal toxicity data reported in the literature. Ideally an exposure period will equal or slightly exceed the average doubling time of the indicator cell. This guarantees a uniform exposure to the test population if there is cell-cycle or cell-division dependence for cytotoxicity. Sensitivity is lost at shorter exposure times because of a decrease in the maximum applied dose. Extension of the exposure time beyond 24 hours creates problems in quantifying cytotoxicity. Long exposure times, especially when coupled with long attachment periods, create the situation of dosing multiple cell colonies instead of single cells. When several cells must be killed to prevent the formation of a colony, there is a decrease in the measured cytotoxicity and alteration of the apparent kinetics of cell killing. Additional problems with long exposures are encountered with unstable or reactive test samples. This type of sample may degrade during the course of exposure, leading to uncertain dose calculations. The range of 16 to 24 hours exposure

provides a practical solution to the problem of defining a standard time of exposure against the need for flexibility in grouping sufficient data for evaluation.

While exposure periods of between 2 hours and 7 days were noted during this literature search, a large body of cytotoxicity data exists in the 16- to 20-hour exposure category. These data were produced as preliminary toxicity studies for various standard point mutation assay systems. For example, literature citations using the CHO-HGPRT assay system developed by Hsie et al. (14) can provide a clonal toxicity data base gathered under standardized conditions. Literature searches such as those provided by the Environmental Mutagen Information Center (EMIC), Oak Ridge, Tennessee, provide a source of current references for applications of standard mutation assays which generally would include clonal toxicity data.

Permanent mammalian cell lines in culture will provide generally similar measures of toxicity to a test agent under standarized test conditions. Variations in sensitivity can occur and perhaps can be minimized by selecting cell lines derived from a common organism for comparison. The Chinese hamster, Cricetulus grieseus, is a logical choice. Chinese-hamster-derived cell lines such as the CHO-K1 (15), CHO-K1-BH_4 (14) and V79 (16) provide ideal characteristics for clonal cytotoxicity measurement. These cell lines generally have a colony-forming potential of 80 to 100 percent in a defined growth medium, a low and relatively stable chromosome number, a doubling time of 11 to 14 hours, and are genetically and biochemically well-characterized (9,17). These cells have the added advantage of permitting the quantitative detection of a variety of altered genetic endpoints (e.g., mutation, chromosome aberrations and sister chromatid exchange [SCE]). Genetic markers are available to detect mutations relating to nutritional requirements, drug resistance or temperature sensitivity. These cells are also compatible with microsome or intact cell metabolic activation systems (9).

Differences in media should have little effect on detecting cytotoxicity assuming that the medium provides optimum culture conditions. Test samples which alter culture medium pH and osmolarity, or which interact with medium components may exhibit variable toxicity with different media. The serum concentration used to supplement the medium could affect assay sensitivity for test substances which react with proteins. Clonal toxicity test systems commonly use between 5 and 10 percent serum. Modifications are possible to decrease serum levels to increase assay sensitivity when testing compounds known to interact with protein (18). Data will be considered for comparison from assays run with up to 15 percent serum.

Variations in cell-plating density or culture-dish size should not affect clonal cytotoxicity measurement. The major considerations are to maintain colony separation during growth and to provide sufficient medium to prevent nutrient depletion. Initial plating densities of between 100 and 500 cells are commonly used. Using less than 100 cells decreases accuracy at higher levels of cell killing. Commonly used plate sizes are 60 x 15 mm and 100 x 20 mm. Practical considerations for counting prevent more than 200 or 500 cells being plated in 60- or 100-mm plates respectively, due to physical crowding of colonies above these levels. Higher

cell densities may also permit metabolic alteration of the test substance or metabolic cooperation between cells, thus affecting the apparent toxicity.

The length of time between cell plating and initiation of treatment can affect apparent toxicity and assay sensitivity. Attachment times vary, but commonly fall between 3 and 24 hours. Three hours generally provide for a firm attachment of newly plated cells to the substrate. Shorter periods may create an artifical sensitivity due to dosing debilitated cells recovering from the trauma of trypsinization. There is a lag period of 15 to 20 hours for cell division to resume in many Chinese-hamster-derived cell lines after quantitative single-cell plating. Attachment periods which exceed the average lag period may serve to underestimate the apparent cytotoxicity and alter the slope of the cell-killing curve. This is due to the formation of two- or multiple-cell colonies prior to dosing, which all must be killed to be detected as inhibition of colony formation. Recommendations for Level 1 clonal toxicity testing currently call for a 6-hour attachment period. Data can probably be safely compared from assay systems using between 3- and 24-hour attachments.

The following list summarizes the standard range of parameters used for selecting toxicity data for comparison of sensitivity. These criteria will permit a uniform base of toxicity data to be collected and quantitatively compared.

TABLE 6

STANDARD RANGE OF PARAMETERS FOR COMPARISON OF CHO TOXICITY DATA

	Parameter	Range
1.	Exposure Period	16 to 24 hours
2.	Cell Lines	Permanent Chinese-hamster-derived cell lines
3.	Medium	No limitation
4.	Serum Concentration	Up to 15%
5.	Plate Size	No limitation
6.	Cell Density	100-200 (60 x 15-mm plate) 100-500 (100 x 20-mm plate)
7.	Attachment Time	3 to 24 hours

A comparison of 31 substances is presented in Table 8. The chemicals are organized by Multimedial Environmental Goals (MEG) categories. This classification scheme was developed as part of the EPA Level 1 environmental assessment testing program. Each entry includes identification of the chemical, the cell line used, the length of exposure time, the estimated EC_{50} and the reference. Also included is the minimum amount of chemical required in a mixture to observe the EC_{50}. This value is reported, not as a chemical concentration, but as a percentage of the maximum applicable dose (MAD) which is 1000 µg/ml for the CHO assay. A summary of the range of toxicity (EC_{50} values) for each chemical class is provided in Table 7.

Cytotoxicity data were often reported graphically in the literature and required interpolation to ascertain EC_{50} values. Results reported on a molar concentration basis are converted to a µg/ml basis where possible. Chemicals are grouped by chemical class and entries include the chemical name, indicator cell, exposure time, the EC50 value, and the minimum concentration in a mixture for which the chemical would give an EC50 value at the MAD dose, and the reference.

TABLE 7

RANGE OF EC_{50}[a] VALUES IN THE LEVEL 1 CHO CLONAL TOXICITY ASSAY SUMMARIZED BY CHEMICAL CLASS

MEG Group[b]	Chemical Class	No. of Entries	Low and High EC_{50} (µg/ml)	Chemical
8	Carboxylic Acids and Derivatives	1	5700	caprolactam
12	Nitrosamines	7	0.5	N-nitrosomethyl-urethane (NMUT)
			10000	dimethylnitros-amine
14	Sulfonic Acids, Sulfoxides	5	4.5	dimethylsulfate (DMS)
			317.9	propylmethane sulfonate (i-PMS)
23	Heterocyclic Nitrogen Compounds	1	1	ICR-191
	Metals and Organometallic Compounds[c]	17	.062	cadmium chloride
			852.7	chromic chloride-hexahydrate

[a] EC_{50} = Effective Concentration of chemical that reduces colony development by 50 percent relative to control levels.

[b] MEG = Multimedia Environmental Goal Chemical classification scheme (4).

[c] Metals and organometallic compounds are summarized as a group and not as individual elements.

TABLE 8

A COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 CHO CLONAL TOXICITY BIOASSAY

MEG Group[a]	Chemical	Cell	Hours Exposure	EC_{50}[b] (µg/ml)	Percent Required To Observe EC_{50} at MAD[c]	Ref.
8	CARBOXYLIC ACIDS AND DERIVATIVES					
	caprolactam	CHO	16	5,700	ND[d]	19
12	NITROSAMINES					
	N-nitrosomethyl-urethane (NMUT)	CHO-K1	16	0.5	0.05	20
	N-ethyl-N-nitro-N-nitrosoguandine (Ethyl-nitrosoguanidine) (ENNG)	V79	16	.258	0.03	21
	N-methyl-N-nitroso-urea (MNU)	V79	16	1.9	0.2	21
	N-nitrosomethyl-urea (NMU)	CHO-K1	16	35	3.5	20
	N-ethyl-N-nitroso-urea (ENU)	V79	16	77.3	7.7	21
	N-butyl-N-nitroso-urea (BNU)	V79	16	145.2	14.5	21
	Dimethylnitros-amine (NDMA)	CHO-K1	16	10,000	ND[d]	20

TABLE 8 (Continued)

A COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 CHO CLONAL TOXICITY BIOASSAY

MEG Group[a]	Chemical	Cell	Hours Exposure	EC_{50}[b] (μg/ml)	Percent Required To Observe EC_{50} at MAD[c]	Ref.
14	SULFONIC ACIDS, SULFOXIDES AND DERIVATIVES					
	methylmethane sulfonate (MMS)	CHO-K1-BH_4	16	6.1	.6	22
	ethylmethane sulfonate (EMS)	CHO-K1-BH_4	16	110.0	11.0	14
	propylmethane sulfonate (i-PMS)	CHO-K1-BH_4	16	317.9	31.8	22
	dimethylsulfate (DMS)	CHO-K1-BH_4	16	4.5	0.5	22
	diethylsulfate (DES)	CHO-K1-BH_4	16	200.5	20.1	22
23	HETEROCYCLIC NITROGEN COMPOUNDS					
	ICR-191	CHO-BH_4	16	1	0.1	23
	METALS AND ORGANOMETALLIC COMPOUNDS[e]					
49	arsenic trioxide	CHO-K1	16	15	1.5	24
32	beryllium chloride	V79	20	199.8	20.0	10
82	cadmium chloride	CHO-K1	16	.062	0.006	24
74	cobalt chloride	V79	20	5.2	0.5	10

TABLE 8 (Continued)

A COMPARISON OF LEVELS OF DETECTABILITY IN THE LEVEL 1 CHO CLONAL TOXICITY BIOASSAY

MEG Group[a]	Chemical	Cell	Hours Exposure	EC_{50}[b] (μg/ml)	Percent Required To Observe EC_{50} at MAD[c]	Ref.
	METALS AND ORGANOMETALLIC COMPOUNDS (Continued)					
74	cobalt chloride-hexahydrate	CHO-K1	16	25.0	2.5	24
68	chromic chloride-hexahydrate	CHO-K1	16	852.7	85.3	24
68	chromic oxide	CHO-K1	16	.5	0.05	24
29	potassium dichromate	V79	24	.76	0.08	25
68	chromic acetate	V79	24	>200	>20	25
81	zinc chromate	V79	24	2.5	0.3	25
46	lead chromate	V79	24	>10	>1	25
71	manganese chloride	V79	20	113.3	11.3	10
71	manganese chloride-tetrahydrate	CHO-K1	16	49.5	5.0	24
76	nickle chloride	V79	20	51.8	5.2	10
76	nickle chloride-hexahydrate	CHO-K1	16	33.3	3.3	24

TABLE 8 (Continued)

A COMPARISON OF LEVELS OF DETECTABILITY IN THE
LEVEL 1 CHO CLONAL TOXICITY BIOASSAY

MEG Group[a]	Chemical	Cell	Hours Exposure	EC_{50}[b] (µg/ml)	Percent Required To Observe EC_{50} at MAD[c]	Ref.
	METALS AND ORGANOMETALLIC COMPOUNDS (Continued)					
46	lead acetate-trihydrate	CHO-K1	16	569	56.9	24
54	selenium dioxide	CHO-K1	16	10.4	1.0	24

[a]MEG = Multimedia Environmental Goals chemical classification scheme (4).

[b]EC_{50} = Effective Concentration of chemical that reduces colony development by 50 percent of control levels.

[c]MAD = Maximum Applicable Dose.

[d]ND = Not detectable with Level 1 test procedures and evaluation criteria.

[e]Metals and organometallic compounds are reported as a group and not as individual elements.

3. Evaluation of Clonal Cytotoxicity Sensitivity

Level 1 bioassays, including the CHO clonal toxicity assay, have inherent levels of sensitivity to detect various classes of chemicals. Lack of observable toxicity up to the MAD (maximum applicable dose) does not rule out the possibility of toxicity at higher doses as seen with caprolactam and dimethylnitrosamine. This section has identified the reported EC_{50} values for various chemicals collected under the standard CHO toxicity assay parameters outlined above. An attempt has been made to predict and compare the level of sensitivity by determining the minimum concentration the chemical could constitute in an environmental mixture and still provide an EC_{50} value at the MAD dose. This exercise, like those for the Ames and Quantal assays, may or may not reflect real-life testing situations because of possible synergistic or antagonistic chemical interactions in complex environmental mixtures.

The CHO cytotoxicity assay has been designed for maximum assay sensitivity. The maximum applicable dose for Level 1 testing has been defined as 24 hours of exposure at a concentration of 1,000 µg/ml, 600 µl/ml or 20 µl/ml for solids, aqueous samples (including slurries or suspensions) or organic vehicle samples respectively. Physical constraints generally prevent any significant increase in MAD concentrations to enhance sensitivity with the possible exception of solids. Solids can be tested up to 10,000 µg/ml but this range is extreme and not recommended for routine Level 1 testing. The exposure time can be increased to enhance sensitivity but, as discussed in the previous section, would produce data not comparable with values generated under the standard protocol.

Situations commonly arise in Level 1 CHO cytotoxicity assays where linear inhibition of colony formation is noted with increasing dose, but does not reach 50-percent killing at the MAD dose level. The purpose of Level 1 testing is served by reporting nondetectable toxicity for such situations. However, estimates of the EC_{50} value can be made and reported by extrapolating linear dose-response curves to the 50-percent level. This is a valid method to enhance assay sensitivity but EC_{50} values determined by data extrapolation should be reported as being best estimates only.

The Table 4 data indicate that the standardized clonal toxicity assay is capable of detecting chemicals with a wide range of toxicities. Based on the limited data base, it is difficult to make definitive statements on assay sensitivity. The toxicity and related limits of detectability can be predicted for some of the simple alkane and alkyl sulfonates based on structure. It can be noted that toxicity for both classes decreased as the size of the substituted alkane or alkyl groups increased. The toxicity of heavy metals varied greatly between metals and between chemical forms. Toxicity and related assay sensitivity would be difficult to predict from the metal alone. Chromium, for example, showed a wide variation in toxicity depending on its chemical form.

Data from this type of evaluation can provide a valuable insight into the capabilities of mammalian <u>in vitro</u> clonal toxicity tests. The term

nondetectable toxicity can take on a quantitative meaning with this type of approach. Unfortunately, the clonal toxicity data base is not as extensive as with the Ames and rodent toxicity tests after the restrictions of a standardized protocol are imposed. Practical application of this technique will require a much larger data base.

E. Level 1 Bioassays Not Reviewed

The limits of resolution and detectability of the remaining Level 1 bioassays have not been addressed in this report. These assays, for the most part, have not been applied on a sufficiently large scale or performed under standardized conditions for any valid comparison of the data to be made. A complete review of the sensitivity of these assays can be made in the future once a larger data base and standard study designs have been developed.

1. Health Effects Assay

The rabbit alevolar macrophage (RAM) assay is the only health effects assay without a suitable data base for evaluation. Numerous complex environmental samples and simulated environmental samples have been tested in this assay using the standard Level 1 protocol by a number of different laboratories. Lacking is an extensive published data base with a wide range of pure chemicals. The prospects are good that, as this assay is incorporated into routine testing programs, additional validation work with pure chemicals will be undertaken and reported.

2. Aquatic Ecological Assays

In aquatic toxicology there is a long record of investigation into the physiologic response of aquatic organisms to specific pure and complex samples. Unfortunately, there is not a large data base for work conducted exclusively with Level 1 protocols using the recommended indicator organisms. It is beyond the scope of this project to compare and interpolate data collected from similar, but basically different, study designs.

Acute freshwater and marine aquatic assays commonly seek to identify the dose (concentration of sample applied for a given length of time) that kills a given number of test organisms. Also, qualitative observations are made of the sublethal behavior and visible physiologic response of the animals to the test compound. In these ways, Level 1 fish and invertebrate assays are similar to the acute toxicity test in rodents. When a sufficient data base is developed, the sensitivity of each assay can be evaluated in the same manner as the rodent test.

Level 1 algal assays use growth inhibition or stimulation as the observed endpoint. The data in this situation resemble the discontinuous data set in the Ames assay. Test samples can produce either positive or negative growth effects which then can be ranked further by toxicity category. This assay, like the Ames assay, provides a distinct dichotomus response which makes the evaluation of assay sensitivity quite easy. Unfortunately, data are scarce on pure chemicals using either the marine or freshwater algal assay.

The following table defines critical assay parameters (test organism and measured end point) for the aquatic ecological assays.

TABLE 9

BASIC PARAMETERS FOR COMPARISON OF
LEVEL 1 STATIC ACUTE AQUATIC BIOASSAY DATA

Assay Type	Test Organism	Measured Effect[a]
Freshwater fish	Pimephales promelas	96-hour LC_{50}
Freshwater invertebrates	Daphnia magna	48-hour EC_{50}
Freshwater algae	Selenastrum capricornutum	120-hour EC_{50}
Marine fish	Cypinodon variegatus	96-hour LC_{50}
Marine invertebrates	Mysidopsis bahia	96-hour EC_{50}
Marine algae	Skeletonema costatum	120-hour EC_{50}

[a]The Maximum Applicable Dose (MAD) in each case is 1 gm solids/L or 100 percent aqueous liquid except for the freshwater algae with a MAD of 80 percent aqueous liquid. See Reference 1 for details.

At the present time, very little data on toxicity of pure substances using the above parameters and Level 1 protocols are available. An analysis of the aquatic testing methods and samples of limited effects with pure samples is provided in "Water Quality Criteria" published in the Federal Register (26). The methods developed for evaluation of sensitivity of CHO and rodent toxicity data applicable to the type of data from the aquatic assays can be used once a suitable data base has been established.

3. Terrestial Ecological Assays

The three Level 1 terrestial ecological assays have not been validated as well as the other Level 1 assays. This lack of published work, both on procedures and assay validation, has prompted the Industrial Environmental Research Laboratory - RTP to direct the production of a laboratory workbook for the terrestrial assays (27). No attempt was made to collect

and evaluate data from these assays. The plant stress ethylene and the root elongation assays are the newest and, therefore, have the least amount of published material.

The Drosophila insect toxicity assay presents a different situation. Existent data cannot be compared because toxicity determinations using Drosophila have not been conducted with anything that approaches a standardized procedure. Another problem is that toxicity in insects may be modified by the palatability of the test material. In all other Level 1 assays, the test organisms are exposed to samples with no option to avoid exposure. This is not true for Drosophila which can avoid feeding on the test material. Assay protocols, therefore, should require use of pre-starved flies; however, this technique offers no assurance that the flies will feed on the test material. Another important factor is test agent solubility or particulate size since Drosophila cannot ingest particles >3μm in diameter. Therefore, compounds which are easily solubilized in water give the most reliable data for comparative purposes.

Thus, at the present time a good estimate of the level of sensitivity of Drosophila to a broad range of chemicals cannot be developed. There is no doubt that this organism is capable of responding to very low doses of certain chemicals such as 1,2-dibromoethane which appears to be more genotoxic in Drosophila than in bacteria, and could be detected at 0.21 ppm (28). However, Drosophila performance with complex mixtures and a broad range of chemical classes must be defined within the context of Level 1 testing as an ongoing process.

IV. CONCLUSIONS

It is possible to estimate the level of sensitivity of bioassays used in Level 1 Environmental Assessment. However, with the exception of only a limited number of tests currently proposed, a data base collected under conditions similar to those recommended for Level 1 testing is not available. For those tests it is advised that the data base be developed during ongoing Level 1 analyses. Where the data bases were available, estimates of the sensitivity levels were made. The mammalian *in vitro* clonal toxicity test and the Ames *Salmonella* test appeared to be quite sensitive compared to the *in vivo* rodent toxicity test. The two *in vitro* tests also approach the sensitivity required of the chemical analyses performed in Level 1 assessments.

Existing data resulting from EPA/IERL-RTP's ongoing Level 1 environmental assessment programs may also provide sufficient chemical and biological data to evaluate the sensitivities of the remaining bioassays.

The information presented in this report might be useful, not only in developing an appreciation of the intrinsic sensitivity of the bioassays, but may also be helpful in modifying assays in order to increase their sensitivities.

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